INVESTIGATION OF STIMULATORY AND INHIBITORY MECHANISMS OF TRANSIENT RECEPTOR POTENTIAL AND PITUITARY ADENYLATE CYCLASE-ACTIVATING POLYPEPTIDE RECEPTORS IN VITRO

PhD THESIS

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2016

1. RESEARCH CONCEPT

Pain is an unpleasant subjective sensory and emotional experience indicating actual or potential tissue damage. Nociceptors are sensory nerve endings reacting specifically to tissue damaging stimuli (thermal, mechanical, chemical), they transmit pain signals to the CNS. Capsaicin acts on the Transient Receptor Potential Vanilloid 1 (TRPV1) receptor, which is localised on a special type of nociceptors also called capsaicin-sensitive peptidergic afferents. Capsaicin-sensitive sensory nerve endings exert triple functions: afferent, local and systemic efferent. Neurogenic inflammation plays an important role in the pathogenesis of several diseases (migraine, asthma, inflammatory bowel diseases, rheumatoid arthritis) and it cannot be influenced by currently available drugs (Szolcsányi et al., 1996). Therefore, this underlines the need to develop novel analgesic and anti-inflammatory drugs, mainly acting on sensory nerve terminals. H_2S is capable of activating capsaicin-sensitive peptidergic afferents, thus may contribute to inflammation and pain. TRPV1 is a major target in pain research, as both antagonists and agonists can exert analgesic effect. Nevertheless, administration of TRPV1 antagonists is limited by severe side effects (hyperthermia, impaired noxious heat sensation). Investigation of hydrophobic interactions of these receptors might be an alternative experimental approach to influence receptor function. Several TRP receptors form signal transduction complexes in lipid rafts, whose structural integrity affect receptor function. The most accepted hypothesis of migraine pathogenesis is the trigeminovascular theory (Moskowitz, 1992). As a result of trigeminovascular activation, vasoactive neuropeptides, such as calcitonin gene-related peptide (CGRP), substance P (SP) and vasoactive intestinal polypeptide (VIP) are released from nerve endings, which induce plasma protein extravasation and mast cell degranulation, establishing neurogenic inflammation. Pituitary adenylate cyclase-activating polypeptide (PACAP) is expressed in several brain regions related to the trigeminal system (Tajti et al., 2001). Plasma PACAP levels were elevated during the ictal phase of migraine and PACAP infusion triggered migraine-like headache in migraineurs (Tuka et al., 2013; Schytz et al., 2009). Our research group provided the first complex experimental data with behavioural, functional and morphological techniques using gene-deleted mice that PACAP plays a pivotal role in migraine-related pathophysiological processes (Markovics et al., 2012). Based on these observations PACAP receptors are considered to be potential therapeutic targets in anti-migraine drug development.

2. INTRODUCTION

2.1 Effect of lipid raft disruption on TRP receptor activation

2.1.1 TRP receptors

Transient Receptor Potential (TRP) receptors are nonselective cation channels, expressed in several tissues and cell types (Pedersen et al., 2005). Based on sequence and structural homology, the TRP family was subdivided into six subfamilies. TRP channels share some structural similarities, they have 6 transmembrane domains and a pore-forming loop located between the fifth and sixth regions. TRP receptors are polymodal channels: they can be activated by different physical (voltage, temperature, mechanical signals) and chemical (both endogenous and exogenous) stimuli (Nilius and Szállási, 2014).

Thermosensitive TRP receptors are in the focus of our work, which might be new targets for novel analgesic and anti-inflammatory drug development.

TRPV1 receptors are expressed in small and medium dorsal root ganglion (DRG) cells, which give rise to thinly myelinated Aδ and unmyelinated C fibers (Tominaga et al. 1998). Besides capsaicin, other vanilloids (resiniferatoxin (RTX), piperine, zingerone) can also activate the receptor. Endogenous activators include anandamide, N-arachidonoyl dopamine and N-oleoyldopamine (Zygmunt et al., 1999, Chu et al. 2003). Furthermore, TRPV1 is activated by heat (> 43 °C) as well as low pH (< 6) (Nilius, 2007). Inflammatory mediators, such as bradykinin, prostaglandins, tumour necrosis factor α (TNF α) and nerve growth factor can sensitize TRPV1. Receptor activation induces Na^+ and Ca^{2+} influx to the cells resulting in action potential generation and sensory neuropeptide release from nerve terminals.

TRPA1 receptors are highly coexpressed with TRPV1 receptors in a proportion of peptidergic afferent Aδ and C fibers. It contains high number (14-18) of ankyrin repeats in the N-terminus (Story et al., 2003). TRPA1 is activated by noxious cold (<17°C), mechanical stimuli, as well as both endogenous and exogenous ligands. Exogenous agonists include allylisothiocyanate (AITC), formaldehyde, allicin and cinnamaldehyde (Bandell et al., 2004; McNamara et al., 2007; Bautista et al., 2005). Furthermore, TRPA1 is activated by endogenous substances, which are released during inflammation, oxidative stress or tissue damage. Physiologically relevant endogenous TRPA1 agonists are the small endogenous gasotransmitters NO and H_2S. (Andersson et al., 2008; 2012). G-protein-coupled receptors (GPCRs), such as bradykinin and proteinase-activated receptor-2 sensitize TRPA1 receptor (Bandell et al., 2004; Dai et al., 2007). The receptor shows divergence in temperature sensitivity, it is heat-sensitive in invertebrates, cold-sensitive in rodents, and temperature-insensitive in primates. (Chen, 2015).

TRPM8 receptor is activated by temperatures below 26°C, menthol, icilin and eucalyptol (Peier et al., 2002; Bautista et al., 2007). Unlike TRPA1 and TRPV1 channels, TRPM8 does not contain any ankyrin-repeat domains in the N-terminal cytoplasmic region. Binding of PIP_2 to TRP domain cause receptor sensitization, but phospholipase C, protein kinase A and C lead to inhibition (Premkumar et al., 2005; Laing and Dhaka, 2016).

TRPM3 receptor is expressed in a wide variety of neuronal and non-neuronal tissues. The neurosteroid pregnenolone sulfate (PS) is the most potent activator of the receptor (Wagner et al., 2008). The presence of the sulphate group and its stereochemical orientation is important for TRPM3 activation (Majeed et al., 2010). Other steroids also activate this ion channel and a new permeation pathway was proposed for TRPM3 (Drews et al., 2014; Grimm et al., 2005). TRPM3 receptor is involved in acute heat sensing (heat activation threshold: 40°C), inflammatory heat hyperalgesia and thus as a potential target for analgesic treatments (Vriens et al., 2011).

2.1.2 Capsaicin-sensitive sensory nerve terminals

Capsaicin-sensitive peptidergic afferents are special types of sensory nerves with three distinct functions: sensory afferent, local and systemic efferent. According to the classical afferent function, these nerves mediate sensory input to the CNS, resulting in nociception. Morover, proinflammatory neuropeptides (CGRP, SP) are released from the activated nerve terminals, triggering neurogenic inflammation (Jancsó et al., 1967). This process is a local efferent function of the sensory nerve endings (Szolcsányi, 1984 a; b). However, from the activated nerve terminals somatostatin is also released, exerting a systemic anti-inflammatory and analgesic effect (systemic efferent or „sensocrine" function) (Szolcsányi, 2004).

2.1.3 Lipid rafts

On the basis of the Singer-Nicolson fluid mosaic model plasma proteins are randomly located in the lipid bilayer. According to a new theory there are microdomains in the plasma membrane whose structure, lipid and protein content differ from other membrane regions. The best-known microdomains are lipid rafts, which are considered as liquid-ordered phase domains, while non-raft regions form the liquid-disordered phase (Rietveld and Simons, 1998). Several physico-chemical properties of lipid rafts differ from parameters of surrounding membrane. Rafts have lower density, they are enriched in saturated lipids, cholesterol, sphingolipids and proteins in comparison with non-raft regions and they show insolubility in the detergent Triton X-100 at 4 °C. Cholesterol is present in both the exo- and endoplasmatic leaflets and plays a pivotal role in the function and structure of rafts (Simons and Ikonen, 1997). Membrane rafts are involved in wide variety of physiological processes. They regulate membrane transport mechanisms and promote molecular interaction in signal transduction pathways (Parton and Richards, 2003). Numerous members of TRP receptor family form signaling complexes in lipid rafts (Liu et al., 2006). Lipid rafts modulate receptor function and signaling pathways in alternative manner therefore, investigation of rafts might be an important topic in pharmacological research. There are several methods to investigate the role of lipid rafts in receptor function. Methyl-β-cyclodextrin (MCD) removes cholesterol from the plasma membrane and cause disruption of lipid rafts. Sphingomyelin can be hydrolysed by sphingomyelinase (SMase), sphingolipid biosynthesis can be inhibited by myriocin (Kobayashi et al., 2006).

2.2 Identification of the target molecule of hydrogen sulfide donors and dimethyl trisulfide (DMTS)

2.2.1 Synthesis and effects of hydrogen sulfide and polysulfides

Hydrogen sulfide (H_2S) is the third known gaseous mediator of the mammalian body besides nitric oxide and carbon monoxide. It is a new member of small gaseous molecules which control intracellular signaling (Wang et al., 2012). H_2S is produced from L-cysteine by cystathionine β-synthase, cystathionine γ-lyase, 3-mercaptopyruvate (3-MST) sulfurtransferase and cysteine aminotransferase. Recent data suggest that H_2S can also be synthesized from D-cysteine by 3-MST and D-amino acid oxidase. Sulfide catabolism in mitochondria is driven primarily by sulfide quinone reductase enzyme. Polysulfides are generated from H_2S in the presence of oxygen (Kimura et al., 2013). H_2S exhibits both pro- and antiinflammatory characteristics depending on the type of inflammation and the concentration of H_2S. In carrageenan-induced arthritis model paw edema was suppressed by H_2S donors (Zanardo et al., 2006). A polysulfide compound diallyl trisulfide inhibited the inflammatory processes of ulcerative colitis by supressing TNF α expression and inhibiting nuclear factor κB (NFκB) activation (Bai et al., 2005).

2.2.2 Molecular targets of hydrogen sulfide and polysulfides

The signaling pathways and mode of action of H_2S are not yet completely understood, but several molecular targets have been identified. The vasodilatory effect of H_2S has been shown to be caused by the activation of the K_{ATP} channel. Indirect activation of T-type calcium channels might mediate the nociceptive effect of H_2S. Numerous experiments suggest that H_2S and polysulfides stimulate the TRPA1 receptor. Ca^{2+} signals were detected after H_2S administration in TRPA1 receptor-expressing Chinese hamster ovary (CHO) cells *in vitro* (Streng et al., 2008). Our research group revealed that CGRP release from sensory nerve terminals caused by TRPA1 receptor activation mediates the vasodilator effect of H_2S (Pozsgai et al., 2012). Inorganic polysulfides induce TRPA1 receptor activation in astrocytes and sensory neurons (Kimura et al., 2013; Hatakeyama et al., 2015). H_2S-induced inflammation is associated with the NFκB signaling pathway (Chattopadhyay et al., 2012).

2.3 Investigation of PACAP receptor agonists and antagonists

2.3.1 Expression and effects of PACAP

PACAP was isolated in 1989 from ovine hypothalamus extracts based on its ability to stimulate cAMP in rat anterior pituitary cells. PACAP is a member of the secretin-glucagon-VIP family and widely distributed in the central and peripheral nervous system and also expressed in non-neuronal tissues (Miyata et al., 1989). In the CNS PACAP expression was found to be the highest in the hypothalamus. Furthermore, PACAP expression was also confirmed in cortex, hippocampus, thalamus, hypophysis (Vaudry et al., 2000). Moreover, PACAP is present in pain pathways, in the dorsal horn of the spinal cord, DRG, trigeminal ganglion (TRG) cells and capsaicin-sensitive peripheral nerve terminals (Moller et al., 1993; Fahrenkrug and Hannibal, 1998). The effects of PACAP in the central and peripheral nervous systems have been widely studied. PACAP exerts neuroprotective effect and it has a crucial role in neuronal differentiation. PACAP also induces vaso- and

bronchodilation, regulates neurotransmitter release as well as influences the gastrointestinal motility and secretion (Vaudry et al., 2000). PACAP plays a regulatory role in nociception, circadian rhythm and thermoregulation (Davis-Taber et al., 2008; Helyes et al., 2007; Hannibal, 2006).

2.3.2 PACAP receptors and ligands

PACAP receptors are GPCRs, the specific PAC1 receptor binds VIP with much less affinity. In contrast, VPAC1 and VPAC2 receptors show similar affinity for VIP and PACAP (Laburthe and Couvineau, 2002). Several isoforms of the PAC1 receptor exist due to alternative splicing, which can generate different signaling pathways (Arimura, 1998). Several agonists and antagonists of the PAC1 and VPAC receptors have been described, which are pharmacological research tools to investigate PACAP receptors. Based on numerous studies, PACAP6-38 is considered to be a PAC1/VPAC2 antagonist in several cell lines, including human neuroblastoma NB-OK-1 cells (Robberecht et al., 1992). Contrarily, we have found that in certain cells and tissues PACAP6-38 behaves as an agonist, instead of inhibiting PACAP1-38-evoked responses, it in fact induces similar effects. Our group described that similarly to PACAP1-38, PACAP6-38 also decreased the electrical-field and chemical stimulation-induced release of CGRP from sensory nerve endings of isolated rat tracheae (Reglődi et al., 2008). Maxadilan is a potent 61 amino acid-containing vasodilator peptide, which specifically activates the PAC1 receptor (Lerner et al., 1991). Maxadilan 65 (M65) has been considered to be a specific PAC1 receptor antagonist. M65 selectively displaced the binding of [^{125}I]PACAP27 in PAC1 receptor-expressing CHO cell line (Uchida et al., 1998). The pharmacodynamic characteristics of VIP6-28 is partial agonism on VPAC1/VPAC2 receptors; therefore it has antagonistic actions in several models (Schuelert and McDougall, 2006). Ala^{11,22,28} VIP is known as a selective VPAC1 agonist with three substitutions at Thr^{11}, Tyr^{22} and Asn^{28} (Nicole et al., 2000). BAY 55-9837 consisting of 31 amino acid residues is a VPAC2-selective agonist that displays high selectivity for VPAC2 in receptor binding assays (Tsutsumi et al., 2002).

2.3.3 The role of PACAP in migraine

The role of PACAP in migraine and other trigeminal sensory functions has been extensively investigated recently, the expression of PACAP in several ‘‘migraine generator” brain regions of humans and animals related to the trigeminal system are well documented (Tajti et al., 2001). PACAP and its receptors have been described on primary sensory neurons of TRGs, peripheral terminals of capsaicin-sensitive sensory nerves and vascular smooth muscle cells (Tajti et al., 1999; Vaudry et al., 2009). Migraine is a sterile neurogenic inflammation of the dura mater (Moskowitz, 1992). Electrical stimulation of the TRG leads to an increased plasma protein extravasation in rats. (Markowitz et al., 1987). PACAP has been proposed as a mediator of trigeminovascular activation which plays a significant role in migraine (Schytz et al., 2010).

3. AIMS

Currently available drugs for the treatment of neuropathic pain and migraine do not provide satisfactory relief in most cases due to a great range of side effects; therefore there is a great need to develop novel anti-inflammatory and analgesic drugs, which could act directly at the level of sensory nerve terminals. Accordingly, in *in vitro* experiments we aimed to reveal pathophysiological mechanisms, identify key mediators and potential drug targets for novel drug development. Our aims were the following:

I. The effect of lipid raft disruption on TRP receptor activation

Previous discoveries on TRP channels described important functional regions on these proteins, but little is known about the potential alteration in receptor function after changes in the microenvironment surrounding them in the plasma membrane. Here we examined the effect of lipid raft disruption on the chemical activation of the thermosensitive TRP receptors in TRG neurons, peripheral nerve terminals and TRP receptor-expressing CHO cells.

II. Identification of the target molecule of hydrogen sulfide donors and DMTS

TRPA1 receptor has recently become a prominent target in pain research. We have gathered knowledge about increasing number of agonists and there are several antagonists under development. The *in vitro* study of H_2S proves its TRPA1 agonist activity. Our aim was to identify the target of DMTS on primary sensory neurons.

III. Investigation of PACAP receptor agonists and antagonists

PACAP has been proposed as a mediator of trigeminovascular activation, which plays a significant role in migraine. Since only limited data are available about TRG neurons, which contribute to the trigeminovascular activation, we aimed at analysing the actions of this peptide and its analogues considered as agonists and antagonists on sensory neural responses *in vitro*. The culture of TRG neurons might be a translational model system in anti-migraine drug development.

4. EXPERIMENTAL MODELS AND METHODS

4.1 Primary cell cultures and cell lines

4.1.1 Primary cultures of TRG neurons

Cultures were made from 1–4-day-old Wistar rat pups and CD1, $TRPA1^{+/+}$, $TRPA1^{-/-}$ mice pups as described elsewhere (Szőke et al., 2000). TRGs were dissected in ice-cold PBS, incubated for 35 min at 37 °C in PBS containing collagenase (Type XI, 1 mg/ml) and then in PBS with deoxyribonuclease I (1000 UN/ml) for 8 min. The ganglia were then rinsed with PBS and dissociated by trituration. TRG cells were plated on poly-D-lysine-coated (100 μg/ml) glass coverslips and grown in a nutrient-supplemented medium in 24-well plates. Cell cultures were maintained at 37 °C in a humid atmosphere with 5% CO_2.

4.1.2 TRPV1, TRPA1 and PACAP receptor-expressing cell lines

CHO cells stably expressing rat TRPV1, human TRPA1and human PAC1, VPAC1 and VPAC2 receptors were used (Sándor et al., 2005; Gaudin et al., 1996; Nicole et al., 2000; Bourgault et al., 2008).

4.2 Methods

4.2.1 Ratiometric technique of $[Ca^{2+}]_i$ measurement with the fluorescent indicator fura-2 AM

Cell cultures were incubated with 1 μM of fluorescent Ca^{2+} indicator dye fura-2 acetoxymethyl ester (fura-2 AM) (30 min at 37 °C). Calcium transients of TRG neurons to introduced by chemicals were examined with an Olympus BX50WI fluorescence microscope. Fluorescent images were taken with a water immersion objective and a digital camera connected to a personal computer. Cells were illuminated alternately at 340 and 380 nm light generated by a monochromator under the control of Axon Imaging Workbench 2.2 software. Emitted light at 510 nm was measured. The R=F340/F380 which was generated by the software was monitored, then processed by the Origin software version 7.0.

4.2.2 Radioactive $^{45}Ca^{2+}$ uptake experiments

Cells were plated in 15 μl cell culture medium onto 72-well miniplate. The following day, cells were washed with calcium-free Hank's solution, then were incubated in 10 μl of the same buffer containing the test solution and 200 μCi/ml $^{45}Ca^{2+}$isotope (1.3 Ci/mM). After washing with ECS the residual buffer was evaporated in 75 °C the retained isotope was collected in 15 μl 0.1% SDS and the radioactivity was measured in 2 ml scintillation liquid in a Packard Tri-Carb 2800 TR scintillation counter. $^{45}Ca^{2+}$isotope retention was presented in count per minute (CPM).

4.2.3 [^{35}S] Guanosine-5'-O-[γ-thio] triphosphate ([^{35}S]GTPγ S)-binding assay

PAC1, VPAC1 and VPAC2 receptor-expressing CHO cells were homogenized and diluted in 50 mM Tris-HCl to obtain appropriate protein content for the assay. The membrane fractions were incubated in Tris-EGTA buffer containing 20 MBq/ 0.05 ml of [^{35}S]GTPγS (0.05 nM) and increasing concentrations of PACAP1-38, PACAP6-38, VIP, VIP6-28, maxadilan, M65, $Ala^{11,22,28}$VIP and BAY 55-9837 (0.001, 0.01, 0.1, 1, 10, 100

nM and 1 μM) tested in the presence of excess GDP (30 μM). Bound and free [^{35}S]GTPγS were separated by vacuum filtration through Whatman GF/B filter. Filters were washed with 5 ml of ice-cold Tris-HCl buffer, and the radioactivity of the dried filters was detected in UltimaGold F scintillation cocktail with a Packard Tricarb 2800TR liquid scintillation counter. Stimulation is given as a percentage of the specific [^{35}S]GTPγS-binding observed in the absence of receptor ligands.

4.2.4 Patch-clamp experiments

The patch-clamp experiments were carried out on TRPA1 receptor-expressing CHO cell suspension using planar patch-clamp technology in the whole-cell configuration with a four channel medium throughput fully automated patch-clamp platform. The voltage protocol for TRPA1 ion channel started with a short (20 ms) -50 mV step, than the potential returned to the -10 mV holding potential for 20 ms. After this, a 60 ms long ramp was applied to 50 mV. The membrane potential then remained at 50 mV for 10 ms before returning to the holding potential of -10 mV. Pulse frequency was approximately 0.2 Hz. External solution was applied for resuspension of cells, for catching and for sealing. For measurements, external solution was changed to recording solution. After the control period test compounds were added in increasing concentrations. Current values were determined at the 50 mV segment during the voltage protocol. Current value was corrected with the control measurement, which served as a baseline. The difference of these two values was accepted as the current through TRPA1 channels.

4.2.5 Measurement of CGRP release by radioimmunoassay (RIA) method from peripheral nerve terminals of isolated rat tracheae

Rats were exsanguinated in deep anaesthesia (sodium thiopenthal 50 mg/kg i.p.). Isolated tracheae were placed into an organ bath to achieve sufficient amount of peptide release and perfused with oxygenated Krebs solution for 60 min (equilibration period) at 37°C. After discontinuation of the flow the solution was changed three times for 8 min to produce prestimulated, stimulated and poststimulated fractions. Chemical activation was performed in the second 8 min period to elicit CGRP release. CGRP concentrations were determined from 200 μl samples of organ fluid of the preparations by means of radioimmunoassay methods developed in our laboratories (Németh et al., 1998). The trachea samples were weighed and CGRP release was calculated as fmol/mg wet tissue.

4.2.6 Fluorescence spectroscopy

For cholesterol depletion in cell membranes 10 mM MCD was dissolved in PBS and added to the cell wells for 45 min at 37 C before laurdan administration. Primary cultures of TRG neurons were incubated with laurdan in 1 μM final concentration for 40 min at 37°C, then we prepared membrane fraction. FL3-2iHR spectrofluorometer was applied to record fluorescence excitation and emission spectra. To quantify the spectral changes, generalised polarisation (GP) was used to determine the emission and excitation spectra.

4.2.7 Filipin flurescence staining

To analyse the cholesterol depletion cells were stained with the cholesterol-binding compound filipin as described earlier in macrophages (Tabas et al., 1994). CHO cells and cultured TRG neurons were treated with 10 mM concentration of MCD for 45 min at 37 °C for cholesterol depletion. Cell cultures were then rinsed with PBS and fixed with 4% paraformaldehyde. Cells were then rinsed with PBS and quenched with 1.5 mg/ml glycine in PBS for 10 min at room temperature. Cells were then incubated for 2 h with 0.05 mg/ml filipin in PBS/10% FBS. Filipin was removed by rinsing cells three times with PBS and analysed by fluorescence microscopy. Micrographs have been generated using an Olympus Fluoview-1000 system.

4.3 Statistical analysis

All data were expressed as means ± SD or ± SEM of at least three independent experiments. Statistical analysis was performed by Student's t-test or one-way ANOVA with Dunnett's post hoc test in the lipid raft disruption experiments and by Student's t-test or one-way ANOVA with Bonferroni post hoc test in PACAP receptor agonists and antagonists analysis. In all cases $p<0.05$ was considered statistically significant. EC_{50} values were determined with GraphPad Prism 5.0 or SigmaPlot software.

5. RESULTS

5.1 Effect of lipid raft disruption on TRP receptor activation

5.1.1 Effect of sphingomyelinase on TRP receptor activation in TRG neurons

A significant concentration dependent decrease in the percent of AITC- and icilin-sensitive cells was observed after SMase incubation. The treatment had no effect on TRPM3 receptor activation.

5.1.2 Effect of sphingomyelinase on voltage-gated calcium channels and the structural integrity of the inner membrane in TRG neurons

SMase had no effect on the response to KCl and it did not influence the peak of the mean fluorescence responses. Treatment had no effect on the proportion of thapsigargin-sensitive cells.

5.1.3 Effect of ceramide and sphingosine on TRP receptor activation in TRG neurons and CHO cells expressing the cloned rat TRPV1 receptor

Incubation with concentrations of 1 and 10 μM ceramide and sphingosine for 60 min at 37 °C did not diminish the capsaicin-evoked response of TRPV1 receptor-expressing CHO cells, as measured by the radioactive $^{45}Ca^{2+}$ uptake method. Neither ceramide nor sphingosine had any effect on the proportion of capsaicin- and AITC-sensitive cells on cultured TRG neurons. Ceramide or sphingosine did not influence the peak of the mean fluorescence responses.

5.1.4 Effect of sphingomyelinase, ceramide and sphingosine on TRP receptor activation in peripheral nerve terminals

SMase significantly and concentration-dependently inhibited the capsaicin-evoked CGRP release. In the case of AITC similar decrease in CGRP release was observed. In these experiments TRPM8 and TRPM3 activation by icilin and PS, respectively did not release sufficient amount of CGRP to measure by RIA. Thapsigargin was not able to induce CGRP release from the nerve endings. Ceramide and sphingosine (10 μM) had no effect on capsaicin- and AITC-evoked CGRP exocytosis.

5.1.5 Effect of MCD on TRP receptor activation in TRG neurons

Significant decrease in the R value and in the percent of AITC-sensitive cells was observed after 10 mM MCD incubation. In the case of icilin the proportion of responding cells and the R value significantly decreased after incubation with 1 mM MCD. 3 mM MCD treatment abolished the Ca^{2+}-influx. Treatment had no effect on TRPM3 receptor activation.

5.1.6 Effect of myriocin on TRP receptor activation in TRG neurons

The proportion of cells responding to AITC and icilin was significantly decreased after overnight myriocin incubation (200 nM, 37 °C). The treatment significantly diminished the AITC- and icilin-evoked fluorescence change. Myriocin treatment had no effect on TRPM3 receptor activation.

5.1.7 Effect of MCD on the ratio of liquid ordered and disordered membrane phases

After MCD incubation the GP_{Em} changes were from 0.46 to 0.35 and GP_{Ex} changes from –0.63 to –0.72 with a clear shoulder. These spectral changes are clear evidence for the transition from liquid ordered to liquid disordered phase after MCD incubation.

5.1.8 Evaluation of the cholesterol depletion after MCD treatment

As compared with control cells, treatment with MCD (10 mM) strongly reduced filipin labelling of the plasma membrane both in TRPV1 receptor-expressing CHO cells and TRG neurons. Cholesterol in control cells was found in both the plasma membrane and in perinuclear compartments. In MCD-treated CHO cells and TRG neurons cholesterol staining was almost abolished.

5.2 Identification of the target molecule of hydrogen sulfide donors and DMTS

5.2.1 Identification of the target molecule of hydrogen sulfide donors in TRG neurons

The applied H_2S donor solutions during the fluorescent $[Ca^{2+}]_i$ measurements were determined by amperometry. The gasotransmitter administration caused a robust Ca^{2+}-influx after a 20-30 sec latency in TRG neurons from $TRPA1^{+/+}$ mice, which indicated ion channel activation. Both H_2S donors increased $[Ca^{2+}]_i$ reproducibly, NaHS sensitive cells were responded to the selective TRPA1 agonist AITC. Neither NaHS nor Na_2S had any effect on TRG neurons obtained from $TRPA1^{-/-}$ mice.

5.2.2 Identification of the target molecule of DMTS in TRG neurons

DMTS-evoked Ca^{2+} influx was detected in the $TRPA1^{+/+}$ mice TRG cells. The dialkyl polysulfide compound increased $[Ca^{2+}]_i$ rapidly and reproducibly. DMTS had no effect on $TRPA1^{-/-}$ mice TRG neurons. The selective TRPA1 antagonist HC-030031 inhibited the DMTS-induced $[Ca^{2+}]_i$ increase.

5.2.3 Effect of DMTS on TRPA1 currents in TRPA1 receptor-expressing CHO cells

DMTS provoked large TRPA1 current at a 100 μM concentration. HC-030031 (30 μM) inhibited the 100 μM DMTS-evoked TRPA1 current. Dose-response curve was obtained with application of fifferent concentration of DMTS in order to study concentration-response relations, AITC served as positive control. EC_{50} value was determined to 18.46 ± 0.31 μM from the Hill plot.

5.3 Investigation of PACAP receptor agonists and antagonists

5.3.1 Effect of PACAP receptor agonists and antagonists on receptor activation in TRG neurons

Slowly increasing $[Ca^{2+}]_i$ characteristic of GPCRs activation was detected in Wistar rat TRG neurons after PACAP1-38 administration. Surprisingly, similar percentages were detected after administration of the same concentration of PAC1/VPAC2 antagonist PACAP6-38. Co-administration of the agonist and antagonist increased the fluorescence in the neurons. TRG neurons were sensitive to PACAP1-27 VIP, BAY 55-9837, maxadilan. Interestingly, Ca^{2+} responses were detected after antagonists (VIP6-28, M65) administration. In contrast, the selective VPAC1 receptor agonist $Ala^{11,22,28}$VIP had no significant effect on $[Ca^{2+}]_i$. There was

no difference in the responsiveness of the TRG cells in calcium-free ECS. Concentrations inducing the maximal responses in rat TRG neurons were tested in CD1 mouse neurons. The mouse TRG neurons were PACAP1-38-, VIP-, BAY 55-9837-sensitive. As in the case of the neuron culture from rats, similar response was detected after PACAP6-38 and M65 administration. There was a detectable Ca^{2+}-influx in the cells after antagonists administration. The selective VPAC1 receptor agonist $Ala^{11,22,28}$VIP had no significant effect on $[Ca^{2+}]_i$.in TRG neurons from mice.

5.3.2 Effect of PACAP receptor agonists and antagonists on receptor activation on PAC1, VPAC1 and VPAC2 receptor-expressing cell lines

On all PACAP receptor-expressing cells the following methods were used: fluorescent $[Ca^{2+}]_i$ measurements, radioactive $^{45}Ca^{2+}$ uptake experiments and $[^{35}S]GTP\gamma S$-binding assay.

PAC1 receptor-expressing cells were sensitive to PACAP1-38 and maxadilan. PACAP6-38 and M65 had no effect on $[Ca^{2+}]_i$ in contrast to the results obtained on TRG neurons. $Ala^{11,22,28}$VIP and BAY55-9837 had no effect on PAC1 cell line. PACAP1-38 and maxadilan caused GPCR activation in contrast with PACAP6-38, VIP6-28 and M65.

VPAC1 receptor-expressing cells responded to PACAP1-38 and $Ala^{11,22,28}$VIP. PACAP6-38, maxadilan and M65 had no effect on $[Ca^{2+}]_i$ in contrast to the results on TRG neurons. The selective VPAC2 receptor-agonist BAY55-9837 had no effect. PACAP1-38, VIP and $Ala^{11,22,28}$VIP induced GPCR activation, but PACAP6-38, VIP6-28 and M65 had no effect.

VPAC2 receptor-expressing cells were sensitive to PACAP1-38 and BAY 55-9837. PACAP6-38, maxadilan, M65 and $Ala^{11,22,28}$VIP had no effect. PACAP1-38, VIP and BAY 55-9837 resulted in GPCR activation, in contrast with PACAP6-38, VIP6-28 and M65.

5.3.3 Radioactive $^{45}Ca^{2+}$ uptake experiments on TRPV1, PAC1, VPAC1 and VPAC2 receptor-expressing CHO cell lines

Capsaicin induced a significant increase of the CPM value on TRPV1 receptor-expressing cell line. There was no significant change in $^{45}Ca^{2+}$ retention in any of the three PACAP receptor-expressing cell lines after PACAP1-38, PACAP6-38 and capsaicin administration.

6. DISCUSSION

6.1 Effect of lipid raft disruption on TRP receptor activation

Disruption of lipid rafts inhibited TRPV1 receptor activation both on trigeminal sensory neurons and TRPV1-transfected cells, as described in our earlier paper (Szőke et al., 2010). The novel finding reported here is that decomposition of lipid rafts significantly and concentration-dependently decreased the opening properties of the TRPA1, TRPM8 but not the TRPM3 cation channel evoked by their respective agonists. The disappeared filipin staining from the plasma membrane of TRG neurons and TRPV1 receptor-expressing CHO cells as well as fluorescence spectroscopy provided evidence for the depletion of cholesterol after MCD treatment. Nevertheless, clarification of their role in the function of TRP channels resulted in controversial observations. In line with our findings depletion of cholesterol with MCD incubation resulted in impaired TRPV1 receptor activation in DRG neurons (Liu et al., 2006). Meanwhile, it had no effect on heat-activation currents of TRPV1-transfected HEK293 cells (Liu et al., 2003). Others reported previously the amplitude of TRPC1 Ca^{2+} currents decreased after MCD treatment (Bergdahl et al., 2003). We found on TRG neurons that lipid raft disruption decreased the icilin-induced Ca^{2+}-influx through TRPM8 channels. According to an earlier observation on mouse TRG neurons and TRPM8 transfected cell line lipid disruption by MCD enhanced the menthol-induced activation of the channel (Morenilla-Palao et al., 2009). With respect of TRPM8 gating icilin is a more potent agonist than menthol and they might act at different allosteric binding sites. In our experiments lipid raft decomposition had no effect on PS-induced TRPM3 activation on native TRG neurons. The special features of the TRPM3 receptor may explain these findings. TRPM3 differs in several aspects from other members of the TRP channels and have several unique features. A specific steroid-binding site on the protein was characterized. Drews and co-workers suggested that TRPM3 assembles with a presently unknown auxiliary protein into a quaternary complex, and in this form the channel is quite strong to resist the lipid raft destruction (Drews et al., 2014). In most experiments we focused on SMase treatment due to its several advantages over the other two compounds: (1) it has a rapid effect, (2) it exerts a localized action on the plasma membrane and (3) its known metabolites can also be investigated providing an opportunity to exclude their interference with the observed responses. SMase treatment affected not only the percentage of responsive cells but decreased the AITC-evoked Ca^{2+}-transients and caused a delay in the response. We have provided evidence that in peripheral nerve terminals SMase can inhibit TRPV1 and TRPA1 activation at lower concentration than in the cell bodies. We were the first to prove that lipid rafts also have a significant role in the receptor activation on sensory nerve terminals too. We described that the enzyme does not influence the activation of voltage-gated calcium channels and its effect is developed only on plasma membrane using KCl and thapsigargin, respectively. The present data also revealed that metabolites of shingomyelin, ceramide and sphingosine, did not influence the TRPA1 and TRPV1 activation. On the basis of all these findings it can be concluded that the lipid membrane environment modulates the function of TRPV1, TRPA1 and TRPM8 channels, which are involved in pain and thermosensation. We suggest that the hydrophobic interactions

between these TRP receptors and lipid raft interfaces influence their opening properties and therefore, targeting this interaction might be a promising tool for drug developmental purposes.

6.2 Identification of the target molecule of hydrogen sulfide donors and DMTS

We have demonstrated that H_2S-application stimulated an increase in $[Ca^{2+}]_i$ in TRG neurons *in vitro* due to activation of TRPA1. Data obtained in *in vitro* systems provided basis for the discussion of *in vivo* results. Topical administration of NaHS on the mouse ear induced vasodilation and increased the cutaneous blood flow, which was reduced in RTX-treated animals as well as by pretreatment with CGRP and NK_1 receptor antagonists. Our results indicates that H_2S activates capsaicin-sensitive sensory nerves acting on TRPA1 receptors and the resultant neurogenic vasodilatation is mediated by the release of vasoactive sensory neuropeptides CGRP and SP via CGRP and NK_1 receptors. Microfluorimetry and electrophysiological experiments revealed that DMTS activates TRPA1 receptors *in vitro*. Our research group investigated the effect of DMTS in mild heat-evoked mechanical hyperalgesia model *in vivo*. DMTS had analgesic effect in this model in $TRPA1^{+/+}$ and $sst_4^{+/+}$ mice. The analgesic effect of DMTS was detected in neither $TRPA1^{-/-}$ nor in $sst_4^{-/-}$ mice. These data suggest that somatostatin released from sensory nerve terminals in response to TRPA1 receptor activation acting on sst_4 receptors might be responsible for the antinociceptive effect. Our *in vitro* results support these conclusions.

6.3 Investigation of PACAP receptor agonists and antagonists

The stimulatory effect of PACAP1-38, PACAP1-27, VIP, maxadilan, BAY55- 9837 was detected. All peptides induced slowly increasing, transient rise in intracellular Ca^{2+} concentration indicating that Ca^{2+} release derived from intracellular stores. Interestingly, PACAP6-38, M65 and VIP6-28, which were described as antagonists in numerous studies and several model systems, show agonistic effect on primary sensory neurons in $[Ca^{2+}]_i$ measurements. The specificity of these fragment peptides was tested on PAC1, VPAC1 and VPAC2 receptor-expressing cell lines. PACAP1-38 administration increased $[Ca^{2+}]_i$ and caused GPCR activation in all PACAP receptor-expressing cell lines, but the respective selective ligands acted as expected based on previous literature data. Since the observed Ca^{2+}-influx under extracellular calcium-free conditions was the same as in the presence of extracellular calcium, we can conclude that signal is coming from mobilization of the intracellular stores as a result of G protein-coupled receptorial mechanisms. Third, we confirmed that the measured Ca^{2+}-influx came from intracellular stores in radioactive $^{45}Ca^{2+}$ uptake experiment, since we could not measure any significant change in $^{45}Ca^{2+}$ retention in all of the three PACAP receptor-expressing cells after PACAP1-38, PACAP6-38 or capsaicin administration. The surprising and virtually contradictory observations with the fragments considered antagonists in other systems have also been found in certain cells and tissues. Recent study has reported that PACAP6-38 acted as a weak partial agonist at 1 μM concentration in Cos-7 cells (Walker et al., 2014). The fragment of maxadilan, M65 is considered as a specific antagonist of PAC1, it was tested in competition binding analysis on PAC1 receptor-expressing CHO cell line (Uchida et al., 1998). Meanwhile, it was reported, that the PAC1 antagonist M65 did not inhibit

PACAP1-38-induced CGRP release from trigeminal nucleus caudalis (TNC). They suggested that the CGRP release is not associated with the three well-known PACAP receptors, thus they suggested the existence of a novel receptor in the trigeminovascular system, which is not yet identified (Jansen-Olesen et al., 2014). Our results clearly support this idea. Stimulation of all PACAP receptors through G_s/G_q proteins activates adenylate cyclase and PLC (Holighaus et al., 2011). PACAP-signaling is complicated because of the several splice variants of PAC1 receptor. The complexity of GPCR signaling is enlarged by the expansive GPCR binding surface of the G-protein, thus it is able to interact with multiple GPCRs simultaneously (Dautzenberg et al., 1999; Holighaus et al., 2011). Furthermore, PACAP receptors can interact with non-GPCRs (Couvineau and Laburthe, 2012 a, b). In view of these facts, we can accept the emerging concept which describes that different agonists can induce distinct active receptor conformational states and different signaling pathways, designated as ‘‘biased agonism” (Kenakin, 2011). The VPAC1 receptor does not play a role in the PACAP signaling in TRG neurons. This conclusion is supported by the lack of significant response after the selective VPAC1 receptor agonist $Ala^{11,22,28}$VIP in these cells. RT-PCR revealed the presence of mRNA for VPAC2 and several splice variants of the PAC1 receptor, but did not detect the presence of VPAC1 receptor RNA in the rat TRG preparation. Immunocytochemistry showed PAC1 and VPAC2 to be present on small-diameter TRG neurons (Chaudhary and Baumann, 2002).

We provided evidence that PACAP receptor ligands considered to be receptor antagonists in other systems act as potent agonists on trigeminal primary neurons *in vitro*. Our results are in agreement with presumptions of other research groups and support the conclusion that presently unknown receptors or splice variants linked to distinct signal transduction pathways might be involved in the trigeminal neural pathway. Further investigations are needed to reveal the precise mechanisms of PACAP-induced trigeminovascular activation and to identify the promiscuous new targets in this system.

7. SUMMARY OF NEW FINDINGS PRESENTED IN THE THESIS

1. We have provided here the first evidence that besides TRPV1 receptors, activation of further two thermosensitive TRP receptors (TRPA1 and TRPM8) are modulated by lipid raft disruption on sensory neurons. The novel findings reported here showed that this modulation of TRPV1 and TRPA1 exists on sensory nerve endings too. However, disruption of lipid rafts had no effect on TRPM3 receptors. Our results suggest that TRPM3 assembles with a presently unknown auxiliary protein into a quaternary complex, and in this form the channel is strong enough to resist the lipid raft destruction. We can conclude that the hydrophobic interactions play an important role in regulation of receptor function. Based on these findings a novel alternative drug development strategy has been suggested. Further investigation of TRP receptors and lipid raft interfaces might be a promising tool for modulation of receptor function and can lead to new drug targets.
2. We identified the target molecule of DMTS in sensory neurons. We provided here the first evidence that DMTS similarly to H_2S donors induces TRPA1 receptor activation on TRG cells and TRPA1 receptor expressing cell lines. Identification of the target molecule is needed to characterize the precise mechanism of action of polisulfide.
3. We verified the stimulatory effect of PACAP1-38, PACAP1-27, VIP, maxadilan, BAY 55-9837 in TRG neurons. We have provided the first evidence that PACAP6-38, M65 and VIP6-28, which were described as antagonists in literature, showed agonistic effect on primary sensory neurons. In contrast with this finding, antagonists had no effect in PACAP receptor expressing CHO cells. The present study revealed that only PAC1 and VPAC2 receptors play a role in the PACAP signaling in TRG cells. Our results support the conclusion that presently unknown binding sites on the receptors or splice variants are expressed in sensory neurons. Our observations might propose the expression of presently unknown receptor in sensory neurons.

We can conclude that endogenous substances, which contribute to pain processes might have drug developmental potential. Furthermore, we emphasize the importance of the investigation of hydrophobic microenvironment. Clarification of the pathomechanism of migraine and neurogenic inflammation and identification of the targets might open promising future perspectives for novel therapies. Our research group and other groups provided evidence in animal and human studies that PACAP plays a pivotal role in migraine-related pathophysiological processes. Our results suggest that PACAP receptor antagonists exert actions different to those on sensory neurons compared to what has been described earlier. Since PACAP plays a key role in trigeminovascular activation based on these results, development of new selective PAC1 and VPAC2 antagonists is needed for anti-migraine therapy.

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9. LIST OF PUBLICATIONS

9.1 Articles related to thesis

Sághy É., Szőke É., Payrits M., Helyes Z., Börzsei R., Erostyák J., Jánosi TZ., Sétáló G. Jr., Szolcsányi J. (2015). *Evidence for the role of lipid rafts and sphingomyelin in Ca^{2+}-gating of Transient Receptor Potential channels in trigeminal sensory neurons and peripheral nerve terminals.* Pharmacol. Res. 100: 101-116.
IF: 4,408

Sághy É., Payrits M., Helyes Z., Reglődi D., Bánki E., Tóth G., Couvineau A., Szőke É. (2015). *Stimulatory effect of pituitary adenylate cyclase-activating polypeptide 6-38, M65 and vasoactive intestinal polypeptide 6-28 on trigeminal sensory neurons.* Neuroscience. 308: 144-156.
IF: 3,357

Hajna Zs., **Sághy É.**, Payrits M., Aubdool A.A., Szőke É., Pozsgai G., Bátai I.Z., Nagy L., Filotás D., Helyes Zs., Brain S.D., Pintér E. *Capsaicin-sensitive sensory nerves mediate the cellular and microvascular effects of H_2S via TRPA1 receptor activation and neuropeptide release.*
Submitted for publication.

Pozsgai G., Payrits M., **Sághy É.**, Sebestyén-Bátai R., Steen E., Szőke É., Solymár M., Garami A., Orvos P., Tálosi L., Helyes Zs., Pintér E. *Analgesic effect of dialkyl polysulfide compound dimethyl trisulfide in mice is mediated by TRPA1 and sst4 receptors*.
Submitted for publication.

9.2 Articles not related to the thesis

Payrits M., **Sághy É.**, Mátyus P., Czompa A., Ludmerczki R., Deme R., Sándor Z., Helyes Zs., Szőke É. (2016). *A novel 3-(4,5-diphenyl-1,3-oxazol-2-yl) propanal oxime compound is a potent Transient Receptor Potential Ankyrin 1 and Vanilloid 1 (TRPA1 and V1) receptor antagonist.* Neuroscience. 324: 151-162.
IF: 3,357

Pohóczky K., Kun J., Szalontai B., Szőke É., **Sághy É.**, Payrits M., Kajtár B., Kovács K., Környei J.L., Garai J., Garami A., Perkecz A., Czeglédi L., Helyes Z. (2016). *Estrogen-dependent up-regulation of TRPA1 and TRPV1 receptor proteins in the rat endometrium.* J. Mol. Endocrinol. 56: 135-149.
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Szánti-Pintér E., Wouters J., Gömöry Á., **Sághy É.**, Szőke É., Helyes Z., Kollár L., Skoda-Földes R. (2015). *Synthesis of novel 13α-18-norandrostane-ferrocene conjugates via homogeneous catalytic methods and their investigation on TRPV1 Receptor Activation*. Steroids. 104: 284-293.
IF: 2,639

Sághy É., Sipos É., Ács P., Bölcskei K., Pohóczky K., Kemény Á., Sándor Z., Szőke É., Sétáló Gy. Jr., Komoly S., Pintér E. *TRPA1 deficiency is protective in cuprizone-induced demyelination – a new target against oligodendrocyte apoptosis.*
Submitted for publication.

Cumulative impact factor of the publications related to the thesis: **7,765**

Cumulative impact factor of all papers: **16,842**

9.3 Abstracts published in cited journals

Szőke É., **Sághy É.**, Bánki E., Kelemen M., Reglődi D., Tóth G., Couvineau A., Helyes Zs. (2013). *Differential actions of pituitary adenylate-cyclase activating polypeptide receptors on sensory neurons and cell lines*. Journal of Neurochemistry. 125: 163.

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9.4 Poster presentations

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9.5 Oral presentations

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Sághy É., Payrits M., Szőke É., Pintér E., Pozsgai G. *A hidrogén-szulfid és a poliszulfidok hatása elsődleges érzőneuronok sejtesttjére és perifériás érzőideg-végződésekre.* Idegtudományi Centrum PhD és TDK Konferencia, Pécs, 2014.

Sághy É., Szőke É., Bánki E., Reglődi D., Tóth G., Couvineau A., Helyes Zs. *A PAC1 receptor antagonista PACAP6-38 agonista hatásai primer érzőneuron sejteken*. III. Pécs-Oklahoma Symposium, Pécs, Magyarország, 2014.

Sághy É., Bölcskei K., Ács P., Komoly S., Perkecz A., Gaszner B., Sipos É., Kemény Á., Szőke É., Sándor Z., Helyes Zs., Pintér E. *Genetic deletion of the Transient Receptor Potential Ankyrin 1 (TRPA1) receptor inhibits cuprizon-induced demyelination in mice*. Neuropeptides, Aberdeen, Skócia, 2015.

10. AKNOWLEDGEMENTS

I would like to gratefully acknowledge the enthusiastic supervision of Dr. Éva Szőke during this work. I thank Prof. Erika Pintér, the head of the Doctoral School who served as an example of professional dedication. I also thank Prof. Zsuzsanna Helyes, who greatly contributed to the success of our experiments with his superior guidance. I would like to express my gratitude Prof. János Szolcsányi who was a role model for me with his commitment to being a researcher. Furthermore, I want to acknowledge my gratitude to Prof. Gábor Pethő for supporting and helping me in the field of graduate education. I would like to thank Dr. Kata Bölcskei and Dr. Gábor Pozsgai for their advices and help. I thank Prof. Dóra Reglődi and Dr. Eszter Bánki who helped in the preparation of the menuscript and Dr. Zoltán Sándor for constructing the TRPV1 and TRPA1 receptor-expressing cell lines. I also thank Prof. János Erostyák and Tibor Zoltán Jánosi for their contribution to fluorescent spectroscopy measurements. I wish to thank our collaborators Dr. Alain Couvineau for providing us PAC1, VPAC1 and VPAC2 receptor-expressing cell lines, Dr. Gábor Tóth for the synthesis of PACAP receptor agonists and antagonists and Dr. György Sétáló. I am also grateful to Dr. László Tálosi and Péter Orvos for their contribution in patch-clamp experiments. I thank to my collegues Krisztina Pohóczky, Maja Payrits and dr. Kata Csekő for their kind help and patience. I also thank the essential and excellent technical assistance to Mrs. Anna Buzási, Cecília Disztl, Anikó Perkecz, Teréz Bagoly and Dóra Ömböli. I thank to Gedeon Richter Talentum Foundation for supporting my PhD studies. I am also grateful to all my colleagues in the Department of Pharmacology and Pharmacotherapy. Finally, I cannot be grateful enough to my family for their support.